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# Desiccation tolerance in developing soybean seeds: The role of stress proteins

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The consistent correlation between desiccation tolerance in orthodox seed tissue and an accumulation of certain "late embryogenesis abundant" (LEA) proteins suggests that these proteins reduce desiccation-induced cellular damage. The aim of the present work was to test this hypothesis. Exogenous abscisic acid (ABA) was used to elevate the level of heat-soluble LEA-like proteins in axes from immature (30 days after flowering; mid-development) seeds of soybean (*Glycine max* [L.] Merrill cv. Chippewa 64). As the LEA-like proteins accumulated in response to ABA, the leakage of all elements after desiccation and subsequent rehydration markedly declined. Both LEA-like protein accumulation and the decline in desiccation-induced electrolyte leakage were apparently dependent on the presence of ABA. Both effects of ABA were inhibited by cycloheximide. Light microscopy revealed a marked effect of the ABA on cellular integrity following desiccation. Osmotic stress also caused a decrease in desiccation-induced electrolyte leakage and stimulated the accumulation of LEA-like proteins. Our data are consistent with the hypothesis that the LEA-like proteins contribute to the increase in desiccation tolerance in response to ABA, and are consistent with a general protective role for these proteins in desiccation tolerance.

*Key words* – Abscisic acid, desiccation tolerance, electrolyte leakage, *Glycine max*, LEA proteins, seeds, soybean, sugars.

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## Introduction

Our agricultural system relies on the ability of orthodox seeds to tolerate desiccation. Such extreme deprivation of water results in irreversible loss of function in most tissues. In contrast, orthodox seeds have mechanisms to prevent damage. These mechanisms include the accumulation of protective components. Two such components are soluble sugars and proteins (Blackman et al. 1991, 1992, Chen and Burris 1990, Koster and Leopold 1988).

In maturing seed tissue, a group of hydrophilic, heat-soluble and abscisic acid (ABA)-responsive proteins among the "late embryogenesis abundant" (LEA) group of proteins accumulates (Blackman et al. 1991, Galau et al. 1987). This family of proteins has been suggested to play a role in desiccation tolerance (Blackman et al. 1991, Close and Chandler 1990, Skriver and Mundy 1990). The accumulation of these proteins is accompanied by the buildup of soluble sugars (Blackman et al. 1992, Koster and Leopold 1988, LePrince et al. 1990). Recent studies with several species of seeds suggest that the presence of neither LEA proteins nor soluble sugars alone is sufficient to confer complete tolerance to desiccation (Blackman et al. 1991, Bradford and Chandler 1992, Farrant et al. 1993). Thus the contribution of one of these components to desiccation tolerance cannot be readily assessed by tissue survival.

A less stringent method of assessing stress-induced cellular injury in plants is to monitor the rate of electrolyte leakage following stress (e.g. Bramlage et al. 1978, Hoekstra et al. 1989, Murray et al. 1989, Sun and Leopold 1993). Here, we report that exogenous ABA applied

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to immature soybean axes results in an increase in LEA-like protein level. This increased level is associated with a decrease in cellular damage following desiccation and rehydration. The decreased cellular damage can be seen by light microscopy and quantified by reduced leakage of electrolytes. The ABA-induced protection against desiccation-induced damage is prevented by the protein synthesis inhibitor, cycloheximide (CHI), and occurs without the accumulation of soluble sugars. Our data are consistent with the hypothesis that the LEA family of proteins contributes to desiccation tolerance in developing seed tissues.

*Abbreviations* – CHI, cycloheximide; ICAP, inductively coupled argon plasma atomic emission spectrophotometer; LEA, late embryogenesis abundant.

## Materials and methods

### Chemicals

All chemicals were purchased from Sigma unless otherwise indicated. Protein assay and gel reagents were purchased from Biorad Laboratories (Hercules, CA, USA).

### Plant material

Soybean plants (*Glycine max* [L.] Merrill cv. Chippewa 64) were grown in a greenhouse as previously described (Obendorf et al. 1983). Developing pods were removed from plants and surface sterilized in 10% (v/v) bleach (0.5% hypochlorite) for 5 min and then rinsed five times in sterile water. Seeds were staged according to the morphological and fresh weight criteria outlined by Saab and Obendorf (1989). Seeds were utilized at 30 days after flowering (DAF) (45% maximum dry weight; 2.5 g $H_2O$ $g^{-1}$ dry weight; axis 80% green; cotyledon 100% green) or 56 DAF (100% maximum dry weight; 1.23 g $H_2O$ $g^{-1}$ dry weight; axis 0% green and cotyledon 75% green; Blackman et al. 1991).

### Culture conditions

Axes were aseptically excised from the seeds and incubated at 25°C for varying times in the dark on 1% (w/v) agar containing 500 μ*M* *cis*, *trans*-ABA added to the medium from a 100 m*M* stock in ethanol. An equal amount of ethanol was added to the agar of control plates. The pH change caused by the added ABA was less than 0.1 pH unit. Mannitol was added in the indicated concentrations to the media prior to autoclaving. For CHI treatment, axes were pretreated for 1 day with 3.5 μ*M* CHI added to the agar from a filter-sterilized 3.5 *M* CHI aqueous stock solution. This pretreatment was necessary to completely inhibit the synthesis of the heat-soluble proteins. After 1 day, axes were transferred to agar containing 3.5 μ*M* CHI with or without 500 μ*M* ABA for 4 days.

### Extraction and analysis of LEA-like proteins and soluble sugars

Heat (80°C)-soluble proteins were extracted from axes as previously described (Blackman et al. 1992). The proteins were electrophoresed on 10 to 18% polyacrylamide gradient gels containing sodium dodecyl sulphate (SDS) (Blackman et al. 1992). Gels were loaded with heat-soluble proteins extracted from equal amounts (85 μg) of total soluble protein. For some experiments, the levels of individual proteins were quantified by densitometry of the Coomassie-stained gels using ovalbumin as standard. In these cases, protein concentration was calculated by dividing the levels of individual heat-soluble proteins (obtained by densitometry) by the total soluble protein (measured spectrophotometrically by the Biorad protein assay [Bradford 1976]). Sucrose and oligosaccharides were extracted and analyzed by gas chromatography (Blackman et al. 1992).

### Desiccation-induced electrolyte leakage

Axes (7–10) were rinsed for 10 min in sterile, deionized water, blotted dry, weighed and equilibrated at 33.5% relative humidity (RH) (maintained by a saturated solution of $MgCl_2$) at 4°C for at least 3 days to a moisture content of 0.072 g $H_2O$ $g^{-1}$ dry weight. Controls were incubated at saturating RH over water for the same time at the same temperature. Whereas LEA-like proteins are synthesized at high moisture contents at room temperature (Blackman et al. 1991), their accumulation is prevented at 4°C (data not shown). After desiccation, axes were immersed in 3 ml of deionized water, and conductivity (μS $cm^{-1}$) of the immersion fluid was monitored at 1-min intervals for 20 min and then at 2- to 4-min intervals for another 20 min with a CDM83 conductivity meter (Radiometer, Copenhagen, Denmark) at 21°C. To determine the total conductivity releasable from the tissue, the tissue plus immersion fluid were frozen on dry ice and thawed at room temperature with gentle agitation. The freeze-thaw cycle was repeated a total of three times. The total conductivity released by the tissue did not differ significantly between freshly excised, control and ABA-treated axis tissue (35.3 ± 2.8, 32.1 ± 3.3 and 29.1 ± 3.0 μS $cm^{-1}$ $axis^{-1}$, respectively). The rate of leakage was fitted by nonlinear least squares method to a single exponent equation of the form:

$$\frac{C_n}{C_0} = 1 - K_1 e^{-K_2 t}$$

(Fig. 1), where $C_n$ is the conductivity (μS $cm^{-1}$) of the solution at time t (min); $C_0$ = the conductivity (μS $cm^{-1}$) present after three freeze-thaw cycles, and $K_1$ (dimensionless) and $K_2$ ($min^{-1}$) are constants. The maximum coefficient of variation observed for $K_2$ (the rate constant

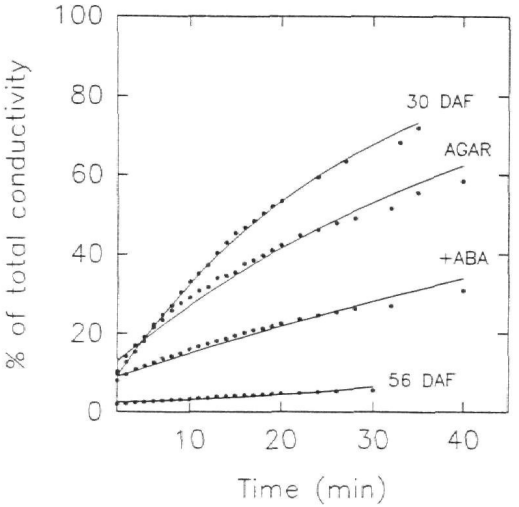


Fig. 1. Time course of electrolyte leakage from 7–10 soybean axes during imbibition of desiccated (0.07 g $H_2O$ $g^{-1}$ dry weight) tissues. Axes from 30-DAF seeds were either desiccated immediately (30 DAF) or treated for 4 days on agar (1% [w/v]) with or without 500 μ*M* ABA (agar and +ABA). Freshly harvested axes from seeds at 56 DAF were desiccated with no further treatment (56 DAF). Leakage data were fitted to the equation described in Materials and methods.

indicating tissue or cellular permeability for electrolytes [Murray et al. 1989]) was 0.7%.

### Leachate analysis

One ml of the immersion fluid was evaporated and the residue was digested in 1 or 2 drops each of nitric and perchloric acid by heating it to 250°C until dryness in quartz tubes. The cooled samples were redissolved in 1 ml of 5% (v/v) HCl and elemental analysis was performed with an inductively coupled argon plasma atomic emission spectrophotometer (ICAP) (model ICAP 61, Thermo Jarrel Ash Corp.).

### Water content determination

The water content was determined by measuring the difference between fresh and dry weights, and expressed as g $H_2O$ $g^{-1}$ dry weight. Dry weights were determined after 24 h at 90°C in a vacuum oven.

### Light microscopy

Axes, either freshly excised or treated with or without 500 μ*M* ABA for 4 days, were either fixed with no further treatment or desiccated to equilibrium with 33.5% RH as previously described and then imbibed in distilled water for 40 min before fixation in 2% (w/v) glutaraldehyde in 50 m*M* Na-cacodylate (pH 7.5) overnight. Tissue was then dehydrated in a graded ethanol series and embedded in methacrylate resin. Two- to 2.5-μm thick sections were stained in 1% (w/v) toluidine blue and viewed with bright-field optics.

## Results

### Association between LEA-like protein accumulation and desiccation tolerance

Soybean seeds are unable to germinate after rapid desiccation to low moisture content until they reach 48 DAF (Blackman et al. 1991). Desiccation-induced electrolyte leakage is a quantitative measure of the extent of stress-induced damage (Bramlage et al. 1978). Thus, desiccation-intolerant tissues should leak electrolytes at a high rate while tolerant tissues should leak electrolytes at a lower rate. Figure 1 shows the leakage of electrolytes from samples of desiccation-intolerant (30 DAF; 45% maximum dry weight) and tolerant (56 DAF; 100% maximum dry weight) soybean axes. By converting the time courses of leakage into rate constants ($K_2$) as in Tab. 1, it is seen that intolerant axes leaked electrolytes at a high rate ($K_2 = 3.5 \times 10^{-2}$ $min^{-1}$) while axes of 56-DAF seeds leaked electrolytes at a 20-fold lower rate ($K_2 = 0.16 \times 10^{-2}$ $min^{-1}$).

We wished to determine if the accumulation of LEA-

Tab. 1. The effect of seed maturation, ABA treatment and desiccation on the rate of electrolyte leakage in isolated soybean axes. Axes were either dried immediately (none) or cultured on agar with (+ABA) or without (−ABA) 500 μ*M* ABA for 4 days. The electrolyte leakage rate constant ($K_2$) was determined as described in Materials and methods and the means ± SD (n = 4) of one representative experiment are shown. ND, Not determined.

| Tissue | Culture | Moisture content before drying (g $H_2O$ $g^{-1}$ DW) | RH during drying (%) | Moisture content after drying (g $H_2O$ $g^{-1}$ DW) | $K_2$ ($\times 10^{-2}$) ($min^{-1}$) |
|---|---|---|---|---|---|
| 30 DAF | None | 2.73 | 100 | 3.34 | 0.08 ± 0.01 |
| | | | 32.5 | 0.13 | 3.47 ± 0.18 |
| | −ABA | 5.95 | 100 | 4.60 | 0.04 ± 0.02 |
| | | | 32.5 | 0.07 | 1.88 ± 0.17 |
| | +ABA | 5.09 | 100 | 4.40 | 0.04 ± 0.02 |
| | | | 32.5 | 0.07 | 0.86 ± 0.08 |
| 56 DAF | None | ND | 32.5 | ND | 0.16 ± 0.02 |
| | +ABA | ND | 32.5 | ND | 0.14 ± 0.02 |

like proteins (which are heat-soluble) might have contributed to this increase in tolerance. The decrease in desiccation-induced electrolyte leakage rate between 30 and 56 DAF was associated with substantial accumulation of nine heat-soluble proteins of molecular weights ($M_r \times 10^{-3}$) of 110, 74, 61, 41, 35, 32, 24, 23 and 22 (Fig. 2, lanes 2 and 4; open and closed circles).

The association between heat-soluble protein accumulation and increased desiccation tolerance observed here led us to suggest (as have others; see e.g. Close and Chandler 1990, Galau et al. 1987, Skriver and Mundy 1990) that LEA proteins contribute to desiccation tolerance. If this is correct, then we can predict that: (1) experimentally induced accumulation of LEA proteins should reduce desiccation-induced leakage and; (2) treatments that impair the ability of tissue to synthesize protein should impair the ability of tissue to show this lessened leakage. In addition, the decrease in desiccation-induced leakage must not be attributable to other factors.

### Effects of ABA on desiccation-induced damage and LEA-like proteins

ABA enhances the expression of *Lea* genes in excised embryos of soybean (Jakobsen et al. 1994). To determine whether alterations in desiccation-induced leakage consistently accompanied ABA-induced changes in LEA-like protein level, we measured the effect of ABA treatment on electrolyte leakage from axes after desiccation (Tab. 1). The rate constant ($K_2$) for electrolyte leakage following desiccation of immature (30 DAF) axes declined 75% after 4 days of ABA treatment while the rate constant for axes incubated without ABA declined only 46%. Despite their lower leakage, axes treated with ABA could not germinate after desiccation (data not shown). In contrast, desiccation-induced leakage from axes from 56-DAF seeds (which were already tolerant to desiccation) did not decline further with ABA treatment (Tab. 1).

Axes were desiccated at 4°C because this temperature prevents the accumulation of proteins that occurs during

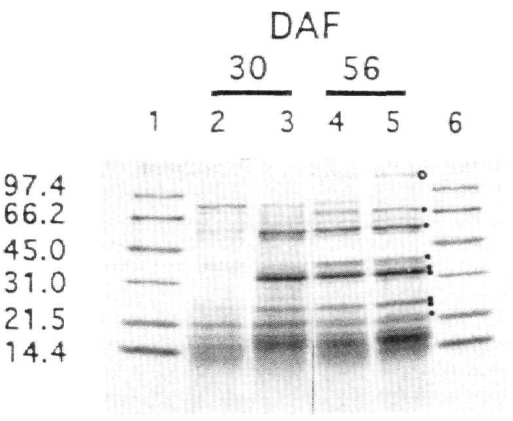


Fig. 2. Effect of seed maturation and ABA treatment on LEA-like protein level in soybean axes. Heat-soluble protein profiles (12–18% SDS-gradient gel) of 30-DAF (lanes 2 and 3) and 56-DAF (lanes 4 and 5) axes treated with (lanes 3 and 5) or without (lanes 2 and 4) ABA for 4 days. $M_r$ ($\times 10^{-3}$) of markers (lanes 1 and 6) are shown on the left. Open circle labels protein that appears during maturation and is not induced by ABA; closed circles label proteins that appear during maturation and are induced by ABA.

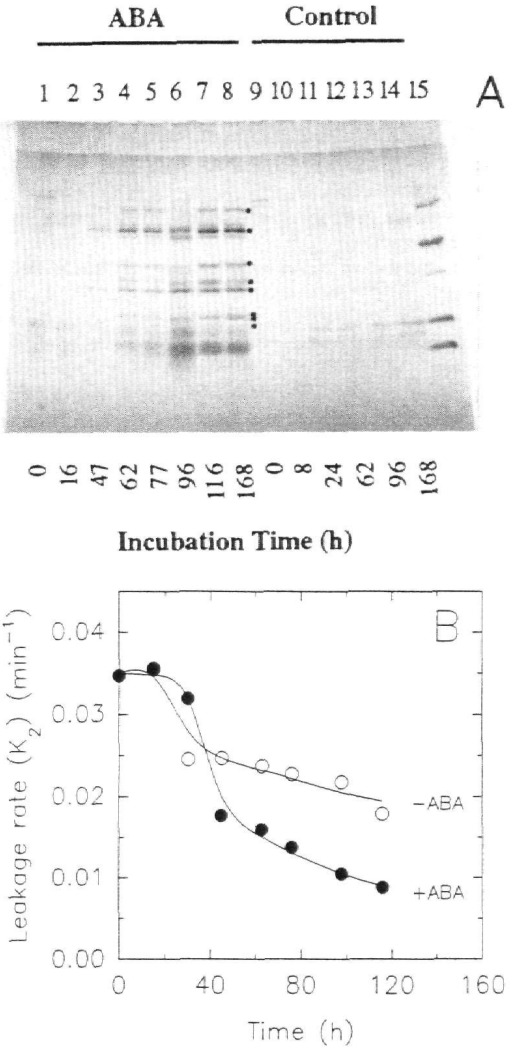


Fig. 3. Time course for desiccation-induced electrolyte leakage and LEA-like protein accumulation in response to ABA. A, Heat-soluble protein profiles (12–18% SDS-gradient gel) of 30-DAF axes with time (h) of treatment with (lanes 1–8) or without (lanes 9–14) ABA. Lane 15 contains $M_r$ markers as in Fig. 2. Circles label proteins that appear during maturation and are induced by ABA. B, Axes from 30-DAF seeds were placed on agar with (+ABA) or without (−ABA) ABA for the indicated time, and then desiccated prior to leakage determination. Values represent the mean of 2 samples.

desiccation at room temperature (data not shown). This procedure leads to possible confusion between damage caused by cold and damage caused by desiccation. To distinguish between damage caused by desiccation and that caused by cold temperature, untreated axes at 30 DAF and axes treated for 4 days on agar with and without ABA were held in water-saturated air (such that they experienced minimal water loss) at the same temperature and for the same time as those that were desiccated (Tab. 1). Following this protocol, axes treated in the absence or presence of ABA had low and almost identical leakage rates ($K_2 = 0.043$ and $0.042 \times 10^{-2}$ $min^{-1}$, respectively). This was approximately 50% of the leakage rate from freshly excised axes subjected to the same high RH treatment ($K_2 = 0.084 \times 10^{-2}$ $min^{-1}$). We can deduce, therefore, that the ABA treatment enhanced the capacity of the axes to withstand desiccation and/or rehydration rather than cold temperature.

The amounts of individual elements that leaked from untreated, ABA-treated and control axes after 20 min imbibition were measured by ICAP. Potassium contributed the greatest share to the conductivity measured in all the leachates. ABA induced a decrease in the leakage of all components (except for sodium) relative to control (axes cultured without ABA) tissue (data not shown). Sodium is normally excluded from the cytoplasm of higher plants (Osmond 1976). Hence, its leakage may not reflect cytoplasmic permeability to electrolytes. These data suggest that ABA decreased the leakage on desiccation of all cytoplasmic electrolytes.

During culture on ABA, axes from 30-DAF seeds (initially 80% green) lost chlorophyll completely whereas axes cultured on agar without ABA retained their green colour. There were no perceptible growth or other morphological changes during the culture period. In contrast, axes of 56-DAF seeds germinated rapidly if they were cultured in the absence of ABA, and consequently, desiccation-induced leakage was not measured. There were also no significant differences in moisture content between ABA-treated and control tissue after any of the experimental protocols (Tab. 1).

Eight of the nine heat-soluble polypeptides that accumulated between 30 and 56 DAF (with $M_r$s [$\times 10^{-3}$] of 74, 61, 41, 35, 32, 24, 23 and 22) accumulated in 30-DAF axes after incubation with ABA (Fig. 2, lanes 2 and 3; closed circles; Fig. 3A, lanes 1 through 8). Axis tissue from 56 DAF seeds, which already contained high levels of these proteins, did not respond further to ABA (Fig. 2, lanes 4 and 5). These results indicate that an increase in heat-soluble proteins induced by exogenous ABA was associated with a decrease in electrolyte leakage following desiccation.

To explore further the correlation between protein level and decreased electrolyte leakage, the time courses for both responses to ABA were examined in detail (Fig. 3). The ABA-responsive proteins began to accumulate after 47 h of incubation on ABA and reached apparent maximal levels after 116 h of incubation (Fig. 3A; closed circles). In contrast, the proteins did not accumulate in the absence of ABA. An ABA-induced decrease in electrolyte leakage was observable after 45 h of incubation on ABA (Fig. 3B), approximately the same time that the ABA-responsive proteins became visible on polyacrylamide gels stained with Coomassie blue. Axes incubated on agar without ABA also showed a decrease in electrolyte leakage, but the magnitude of this decline was not as great as it was with ABA.

Substantial leakage from desiccated tissue can result from rapid hydration rather than damage incurred during desiccation per se. This "imbibitional damage" is prevented if the tissue is humidified prior to imbibition (Bramlage et al. 1978). But the leakage from desiccated ABA-treated tissue was consistently less than that from control, whether or not tissue was humidified prior to imbibition (data not shown). Therefore, the ABA-inducible proteins (or some other ABA-induced factors) apparently ameliorate desiccation-induced damage rather than imbibitional damage.

In summary, we see a strong inverse correlation between desiccation-induced electrolyte leakage and ABA-induced LEA-like protein content in immature soybean axes during culture on ABA.

### Effect of osmotic stress on electrolyte leakage and LEA-like protein level

Osmotic stress as well as ABA is reported to stimulate the accumulation of LEA proteins in plant tissues (Skriver and Mundy 1990). The effects of osmotic stress imposed by mannitol on LEA-like protein formation and electrolyte leakage were therefore examined. The amounts of the eight ABA-inducible heat-soluble proteins in axes incubated at different osmotic potentials were quantified by densitometry of the Coomassie-stained gels. Immature axes treated with 50 g $l^{-1}$ mannitol for 4 days had LEA-like protein contents (0.4% of total soluble protein) and leakage rates following desiccation ($K_2 = 1.9 \times 10^{-2}$ $min^{-1}$) that were similar to those of control (Fig. 4). In contrast, mannitol at 100 g $l^{-1}$ stimulated the accumulation of these proteins 11.5-fold (to approximately 4.6% of the total soluble protein; Fig. 4A) and reduced leakage following desiccation by approximately 50% (Fig. 4B). The effects of mannitol and ABA on LEA-like protein content were additive. That is, LEA-like protein content was higher in axes treated with ABA plus 100 g $l^{-1}$ mannitol than in tissue treated with either 100 g $l^{-1}$ mannitol or ABA alone (Fig. 4A). However, this accumulation of LEA-like proteins did not result in a corresponding decrease in electrolyte leakage following desiccation (Fig. 4B). The lack of enhanced protection at heat-soluble protein levels greater than 2.9% of the total soluble proteins may indicate a saturation of the protective effect.

In summary, both ABA and osmotic stress stimulated the accumulation of eight heat-soluble proteins and induced a concomitant decrease in electrolyte leakage upon rehydration after desiccation. These data are consistent

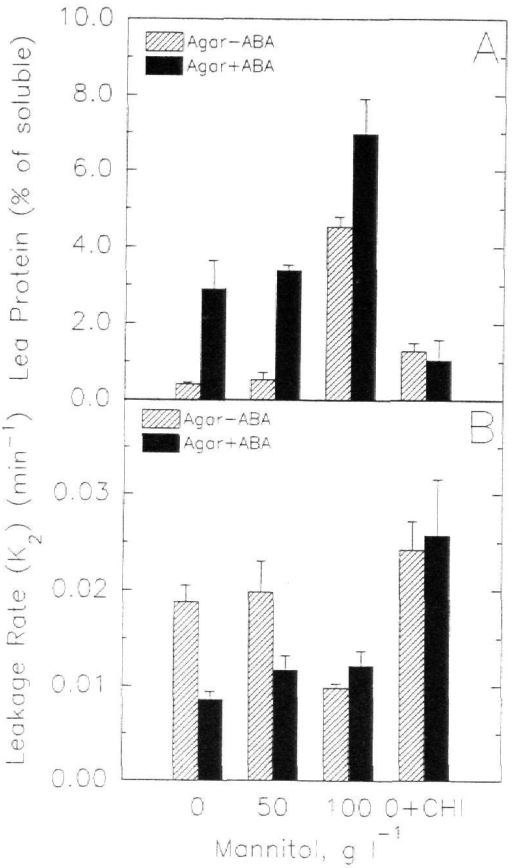


Fig. 4. LEA-like protein accumulation and desiccation-induced electrolyte leakage rate of axes incubated with mannitol or CHI and ABA. A. Heat-soluble LEA protein accumulation expressed as percent of the total soluble protein extracted from axes after incubation for 4 days on media containing 0, 50 or 100 g $l^{-1}$ mannitol in the presence or absence of 500 μ*M* ABA. Axes incubated with CHI were pre-incubated for 1 day on media containing 3.5 μ*M* CHI prior to transfer to medium containing CHI with or without 500 μ*M* ABA for 4 days. The eight heat-soluble proteins marked by closed circles in Fig. 2 and 3A were quantified by densitometry of Coomassie-stained gels. B. Axes were treated with mannitol or CHI ± ABA as described above prior to desiccation and electrolyte leakage measurement (expressed as leakage rate [$K_2$]). All axes were from 30-DAF seeds.

with the proteins being causative agents for the reduction in desiccation-induced leakage.

### Effect of inhibition of protein synthesis

To ascertain whether the ABA-induced reduction in electrolyte leakage following desiccation required protein synthesis, we incubated axes with the protein synthesis inhibitor, CHI (at 3.5 μ*M* for 5 days), in the presence and absence of ABA. CHI prevented both the ABA-induced accumulation of LEA-like proteins (Fig. 4A) and the reduction in electrolyte leakage following desiccation (Fig. 4B). These data suggest that the ABA-induced reduction in electrolyte leakage following desiccation required the synthesis of certain proteins; the LEA-like proteins are candidates for those proteins.

Tab. 2. The effect of ABA treatment (500 μ*M*; 4 days) on the amount of the soluble sugars, sucrose and stachyose in immature axes of seeds of 30 DAF. ND, Not detected.

| Treatment | Sucrose (μg $axis^{-1}$) | Stachyose (μg $axis^{-1}$) |
|---|---|---|
| None | 42.9 ± 8.9 | ND |
| Agar (4 days) | 7.0 ± 3.0 | ND |
| ABA (4 days) | 5.6 ± 4.3 | 2.8 ± 5.8 |

### Effect of ABA on soluble sugars

Desiccation tolerance in many tissues can be partly attributed to soluble sugars (Blackman et al. 1992, Chen and Burris 1990, Hoekstra et al. 1989, Koster and Leopold 1988). But the amount of sucrose in soybean axes decreased substantially during incubation on ABA, when desiccation tolerance was increasing (Tab. 2). Stachyose was not detectable in freshly excised axes, and was barely detectable only in some samples after incubation on ABA. These sugar contents did not differ significantly from those of control axes incubated on 1% (w/v) agar. Thus, the effect of ABA in reducing electrolyte leakage following desiccation cannot be attributed to an increase in soluble sugars.

### Cellular changes associated with ABA treatment and desiccation

The above data are consistent with the hypothesis that ABA- and osmotic-stress-induced heat-soluble proteins reduce desiccation-induced damage in developing axes. We asked whether this reduction in damage was associated with cellular changes visible by light microscopy.

At 30 DAF, axis cells have several obvious vacuoles of varying size and numerous plastids (Fig. 5A). After incubation on agar for 4 days, the vacuolar volume increased and the number of starch-containing plastids decreased (Fig. 5B). Axes treated with ABA for 4 days were similar to control axes save for a further reduction in the number of starch-containing plastids (Fig. 5C). Neither ABA-treated nor control axes acquired the anatomical traits characteristic of germinated tissues (most notably, large central vacuoles) (Crevecoeur et al. 1976).

However, axes cultured in the presence and absence of ABA differed markedly in their responses to desiccation and rehydration. Cells of axes cultured without ABA were destroyed by desiccation and subsequent rehydration (Fig. 5D). Most cells appeared to lack protoplasm altogether, but where it was distinguishable, membranes were vesiculated and cytoplasmic contents were coagu-

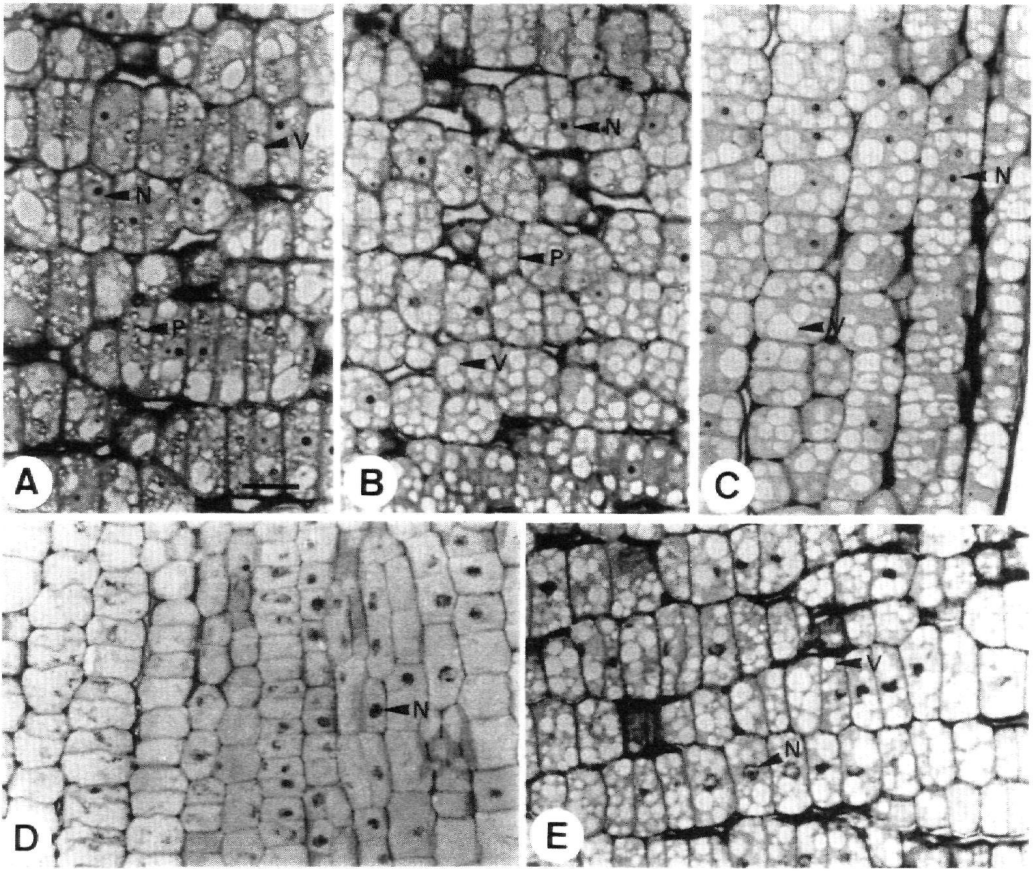


Fig. 5. Cellular anatomy and response to desiccation of immature soybean axes. All light micrographs illustrate longitudinal sections through the cortical cells of axis tissue from a 30-DAF seed. A, Freshly excised axis showing nucleus (N), vacuole (V) and plastids (P). Bar represents 20 μm with all micrographs at the same magnification. B, Axis incubated on agar without ABA for 4 days. C, Axis incubated on agar with 500 μ*M* ABA for 4 days. D, Axis treated as in (B), and then desiccated and imbibed for 40 min prior to fixation. E, Axis incubated as in (C), and then desiccated and imbibed for 40 min prior to fixation.

lated. The nucleus was the only organelle to retain a degree of integrity with the chromatin appearing condensed around the periphery. In contrast to control axes, axes treated with ABA suffered much less visible damage after desiccation and rehydration (Fig. 5E). The protoplasm and vacuoles remained remarkably intact. Although the cytoplasm of ABA-treated axes largely retained its structure after desiccation, chromatin was condensed in the nuclei just as it was in control tissue.

Taken together, our data suggest that the ABA-responsive LEA-like proteins we examined are associated with enhanced stability in cytoplasmic structure in the face of severe desiccation.

## Discussion

Numerous authors have suggested that some of the heat-soluble ABA-responsive proteins which accumulate during seed maturation play a role in desiccation tolerance (see, e.g. Baker et al. 1988, Blackman et al. 1991, Close and Chandler 1990, Close et al. 1989, Dure et al. 1989, Galau et al. 1987, Skriver and Mundy 1990). The basis for this suggestion has been the frequently observed correlation between high levels of gene expression or protein accumulation and desiccation tolerance. However, this association has been found to break down in some recalcitrant seeds (Bradford and Chandler 1992) or when these proteins are prematurely induced in orthodox seed tissues (Blackman et al. 1991).

The set of proteins that accumulate late in embryogenesis includes several well-characterized proteins of known sequence called LEA proteins (Baker et al. 1988, Close and Chandler 1990, Jakobsen et al. 1994, Skriver and Mundy 1990). Although the primary sequences of the soybean proteins examined here have not been deter-

mined, they seem likely to include the LEA group of proteins because they share a number of properties with known LEA proteins: solubility at high temperatures (Jacobsen and Shaw 1989); accumulation late in embryogenesis, and degradation early in germination (Blackman et al. 1991, Goldberg et al. 1989); and enhanced expression in immature embryos in response to exogenous ABA or osmotic stress (Jakobsen et al. 1994, Skriver and Mundy 1990).

The aim of the present work was to test the limits of the association between the accumulation of LEA-like proteins and desiccation tolerance. This was first done by using ABA or osmotic stress as tools to increase the amount of the LEA-like proteins, and by using electrolyte leakage as an assay for cellular damage. In the presence of ABA or osmotic stress, excised, immature soybean axes (30 DAF; mid-development) accumulated LEA-like proteins in amounts similar to those in mature seeds. Reduction in desiccation-induced electrolyte leakage occurred with this accumulation of LEA-like proteins to about 3% of total soluble protein. The effects of ABA on both desiccation-induced electrolyte leakage and heat-soluble protein accumulation were prevented by CHI.

Soluble sugars were not responsible for the ABA-induced desiccation tolerance, even though they make an important contribution to desiccation tolerance during seed maturation in vivo (Blackman et al. 1992, LePrince et al. 1990). Levels of both sucrose and stachyose remained low in axes either in the presence or absence of ABA. Stachyose and sucrose levels of 250 and 75 μg $axis^{-1}$, respectively, were required before a degree of desiccation tolerance (i.e. survival after drying) was achieved in maturing soybean axes (Blackman et al. 1992).

Electrolyte leakage has been used to indicate plasma membrane integrity following stress (Bramlage et al. 1978, Crowe et al. 1989). However, we suggest that the plasma membrane may not be the primary site of action of the ABA-responsive LEA-like proteins for the following reasons. First, many of the LEA family of proteins reside in the cytoplasm or nucleoplasm (Close et al. 1993, Mundy and Chua 1988, Roberts et al. 1993) and, in some cases, the chloroplast (Schneider et al. 1993). Second, leakage from immature axes cultured with ABA (which contained high amounts of the LEA-like proteins) was much higher than leakage from mature, desiccation-tolerant tissue (Fig. 1, Tab. 1). The high rate of electrolyte leakage suggests that the desiccated and rehydrated membrane is no longer intact despite the presence of the LEA-like proteins.

The ABA-induced reduction in cellular damage caused by desiccation was evidenced not only by reduced electrolyte leakage but also by light microscopy. Despite the apparent loss of membrane function, the protoplasm of the ABA-treated tissue still retained its integrity (Fig. 5D,E). Soybean axes cultured in the absence of ABA showed visible cytological damage in response to a desiccation/rehydration cycle. Similar damage occurs in desiccation-intolerant maize embryos where cytoplasmic organelles are unrecognizable and chromatin remains aggregated (Crevecoeur et al. 1976). Axes cultured in the presence of ABA retained protoplasmic integrity but their chromatin remained aggregated. Organelles in fully desiccation-tolerant maize embryos also remain intact but their chromatin decondenses following rehydration (Crevecoeur et al. 1976). Thus, the response of the ABA-treated axes to desiccation and rehydration can be considered intermediate between fully desiccation-tolerant and intolerant tissue both in terms of their cytological structure and their electrolyte leakage rates.

Taken together, our data are consistent with the model that the ABA-responsive LEA-like proteins enhance the structural stability of the cytoplasm. Our data are not consistent with the suggestion that ABA elevates the ability of tissues to retain water (Reynolds and Bewley 1993) since the water contents of both ABA-treated and control tissues were not significantly different when they were equilibrated at 33.5% RH. ABA- and osmotic-stress-induced factors did not confer complete desiccation tolerance. Other factor(s) must be necessary, especially to preserve membrane function (Blackman et al. 1991, Bradford and Chandler 1992). These would include soluble sugars which play a vital role in desiccation tolerance (Blackman et al. 1992, Chen and Burris 1990, Koster and Leopold 1988, LePrince et al. 1990, Sun and Leopold 1993) possibly by enhancing membrane stability (Crowe et al. 1989, Hoekstra et al. 1989).

It is possible that some factor(s) besides the LEA-like proteins is responsible for the protective effects of ABA and osmotic stress that we observed. However, several findings argue against this: (1) the time-courses for the development of desiccation tolerance and accumulation of the LEA-like proteins are similar; (2) the protein synthesis inhibitor, CHI, simultaneously inhibited the accumulation of LEA protein and the reduction in cell damage; (3) osmotic stress only inhibited electrolyte leakage at levels that induced the accumulation of the ABA-responsive LEA-like proteins; (4) the ABA treatment did not induce the accumulation of soluble sugars. Although our data are consistent with the involvement of LEA-like proteins, we cannot rule out other agents being involved in the protection against desiccation-induced electrolyte leakage. It is also possible that the enhancement of cytoplasmic stability that we observed is not the primary role of these proteins, but is ancillary to another.

In conclusion, we have shown that the development of some degree of desiccation tolerance in immature soybeans is closely correlated with the accumulation of a set of LEA-like proteins when they were induced by ABA or osmotic stress. The ABA-induced accumulation of proteins and reduction in desiccation-induced damage was not associated with significant changes in soluble sugars, and both effects could be blocked with CHI. Our data are consistent with the model that the ABA-responsive LEA-like proteins specifically aid in the development of desiccation tolerance in soybean seeds.

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